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Human Th17 Cells Produce a Soluble Mediator that Increases Podocyte Motility via Signalling Pathways that Mimic PAR-1 Activation

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Running Headline: Th17 cells signal to podocytes via the PAR-1 surface receptor

Author Contributions: Moin Saleem, Gavin Welsh, Carl May and Richard Lee conceived of the presented idea. Philippa Lait and Lauren Shewitz-Bowers generated and cultured the Th0 and Th17 cells and hence provided the cell culture supernatants. Carl May performed the experiments with assistance from Musleeha Chesor. Carl May took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research All authors discussed the results and contributed to the final manuscript.

Abstract

The specific pathogenesis of idiopathic nephrotic syndrome (NS) is poorly understood and the role of immune mediators remains contentious. However, there is good evidence for the role of a circulating factor, and we recently postulated circulating proteases as candidate factors. Immunosuppressive therapy with glucocorticoids (GCs) and T cell inhibitors are widely used in the clinical treatment of NS. Given that T helper ($CD4^+$) cells expressing IL-17A (so-called Th17 cells) have recently been reported to be resistant to GC treatment, and GC resistance remains a major challenge in the management of NS, we hypothesised that Th17 cells produce a circulating factor that is capable of signalling to the podocyte and inducing deleterious phenotypic changes. To test this, we generated human Th17 cells from healthy volunteers and added the supernatants from these T-cell cultures to conditionally immortalised human podocytes *in vitro*. This demonstrated that podocytes treated with Th17 cell culture supernatant, as well as with patient disease plasma, show significant

stimulation of JNK and p38 MAPK pathways and an increase in motility which was blocked using a JNK inhibitor. We have previously shown that nephrotic plasma elicits a podocyte response via the protease receptor PAR-1. Stimulation of PAR-1 in podocytes elicited the same signalling response as Th17 cell culture supernatant treatment. Equally, protease inhibitors in the Th17 cell culture treatment blocked the signalling response. This was neither replicated by the reagents added to Th17 cell cultures nor by IL-17A. Hence, we conclude that an undefined soluble mediator produced by Th17 cells mimics the deleterious effect of PAR-1 activation *in vitro*. Given the association between pathogenic subsets of Th17 cells and GC resistance, these observations have potential therapeutic relevance for patients with NS.

Key Words: Th17 Podocyte PAR-1 Circulating Factor Nephrotic Syndrome

Introduction

Nephrotic Syndrome (NS) is defined by the triad of nephrotic range proteinuria, hypoalbuminemia and oedema. As such, structural and functional defects in the glomerular filtration barrier result in an inability to restrict urinary protein loss [7]. While the specific pathogenesis of idiopathic NS is not fully understood, podocytes are widely regarded to be the target cell as they contribute to both the final layer of the glomerular filtration barrier and also to the formation of the glomerular basement membrane [1]. Further support for their role is evidenced by ultrastructural changes seen histologically in podocytes in NS that render them unable to restrict urinary protein loss [5]. This process of ultrastructural change is termed foot process effacement whereby the foot processes retract and the nature of the slit diaphragm that bridges the gap between neighbouring foot processes is changed. This is considered a migratory event and contributes to the overall measure of podocyte motility [19]. Focal Adhesion Kinase (FAK) is a tyrosine kinase that plays a critical role in regulating cell motility, and podocyte specific deletion of FAK leads to a decrease in cell spreading and motility [17]. Notably, mice with this genotype were protected against proteinuria and foot process effacement following podocyte injury [17] and therefore podocyte motility *in vitro* can be used as a surrogate marker for podocyte foot process effacement *in vivo* [13].

NS patients are commonly treated with immunosuppressive glucocorticoids (GCs), which are thought to interfere with production of an immune cell derived circulating soluble factor(s), but which have also been reported to have direct effects on podocytes [21, 22]. Efficacious treatment with GCs is partially determined by the form of NS, in particular whether it is genetic or non-genetic (the former results in GC resistance while the latter can be GC sensitive, GC resistant and interestingly can also be initially GC sensitive and become GC resistant). The efficacy of GCs also varies in inflammatory diseases affecting the lungs, gut and central nervous system, and recent reports have highlighted the role of cytokines expressed by T helper (Th, $CD4^+$) cells in defining GC responsiveness in these conditions, with the co-expression of interferon(IFN)-γ and interleukin(IL)-17A in Th17 cells being characteristic of GC resistance. Similarly, it has been suggested that a bias towards Th17 cells correlates with increased proteinuria in minimal change NS (MCNS) [16].

Given the evidence to support the role of a circulating factor produced by cells of the immune system in the development of non-genetic NS , we speculated that $CD4^+$ T helper cells are a candidate source of such a soluble mediator. Moreover, this hypothesis provides scope for a Th17 driven mechanism of GC resistance in NS (ie, a subset of Th17 cells will not be suppressed in GC resistant patients and GC treatment itself may provide a selection pressure to enrich these GC resistant Th17 cells). Therefore, any factors released into the circulation by Th17 cells, and which are capable of damaging the podocyte, will continue to do so despite GC therapy. We have previously suggested that the unknown factor is a protease which signals via Protease activated receptor-1 (PAR-1) as plasma obtained from post-transplant NS patients during relapse, but not in remission, signals via PAR-1 to increase podocyte motility [8]. VASP was a key phosphorylation target in this signalling response and thought to be responsible for mediating the increase in podocyte motility seen following relapse nephrotic plasma treatment. Hence, in this study we sought to interrogate whether soluble mediators from human Th17 cell cultures were capable of inducing the same signalling pathways and podocyte responses *in vitro* as PAR-1 activation, our goal being to establish whether Th17 cells are a potential source of a circulating factor that can both injure the podocyte and mediate GC resistance in NS.

Methods

Cell Isolation and fluorescence-activated cell sorting (FACS)

Approximately 80mls of whole blood was collected from healthy volunteers following informed consent in accordance with a National Health Service Research Ethic Committee approved protocol at University Hospitals Bristol NHS Foundation Trust, UK (04/Q2002/84). $CD4^+$ T cells were enriched by negative selection using the RosetteSep® Human $CD4^+$ T cell Enrichment Cocktail (Stemcell Technologies, Canada) according to manufacturer's instructions and then washed in wash buffer (PBS supplemented with 1% Fetal calf serum, FCS) and stained with antibodies for 30 minutes at 4°C using anti-CD4 (1:50 dilution; clone OKT-4; eBioscience), anti-CD3 (1:100 dilution; clone UCHT1; BD Bioscience) and biotinylated anti-CCR6 (CD196; 1:50 dilution; clone 11A9; BD Bioscience)/ streptavidin-APC (1:200 dilution, BD Bioscience). 7-Aminoactinomycine D (1:400 dilution; Life Technologies, UK) was used to discern living cells. Cells were then FACS into living $CD4^+CCR6^-$ and $CD4^+CCR6^+$ cells (BD Influx™, BD Bioscience)

Cell culture and supernatant generation

After FACS, cells were re-suspended to $2x10^6$ cells/ml in complete RPMI-1640 (Invitrogen) supplemented with 10% (v/v) FCS, L-glutamine and Penicillin/streptomycin (Pen/strep) (all PAA Laboratories Ltd, UK) and stimulated with plate-bound 5 µg/ml anti-CD3 and 5 µg/ml anti-CD28 antibodies (eBioscience, UK). $CD4^+CCR6^-$ cells did not receive supplementary cytokines. These cells were expected to express IFN-γ on anti-CD3 / anti-CD28 stimulation and were designated Th0. In contrast, $CD4^+CCR6^+$ cells received a polarizing cytokine cocktail of 20 ng/ml IL-6, 10 ng/ml IL-23, 10 ng/ml IL-1β (R&D Systems, UK), 100 ng/ml anti-IFN-γ, 100 ng/ml anti-IL-4 (eBioscience, UK) to generate dual IL-17 and IFN-γ expression for 3 days (designated Th17). Thereafter, cells were transferred to fresh plates and cultured in complete RPMI-1640 containing 50 ng/ml IL-2 only (for Th0) or 50 ng/ml IL-2 plus the polarizing cytokine cocktail (for Th17) for 4 days. This 7-day process was repeated once, and cell supernatants were collected at day 14 and frozen.

Flow Cytometry

Intracellular cytokine production from Th17 and Th0 cells was assessed at day 14. Briefly, cells were incubated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 µM ionomycin and 2 µM protein transport inhibitor (Monensin; BD Bioscience) for 4 hours at 37°C. After

stimulation, cells were fixed, permeabilized (Cytofix/perm solution; BD Bioscience) and stained with anti-IL-17 (1:50 dilution; clone eBio63CAP17; eBioscience), and anti-IFN-γ (1:50; clone 4S.B3; eBioscience). All samples were acquired using a BD LSR II™ (BD Bioscience) and data analysis was run using FlowJo 7.6 software (Treestar).

Podocyte Cell Culture

Conditionally immortalised wild-type human podocytes were cultured in whole podocyte medium : RPMI 1640 supplemented with Insulin Transferrin Selenium (ITS), Pen/strep and 10% (v/v) FCS at the permissive temperature of 33°C until confluent [20]. Subsequently they were switched to the non-permissive temperature of 37°C until they began to differentiate, as previously described [18]. Podocytes were cultured for ten to fourteen days at the non-permissive temperature before being used experimentally.

Treatment and Generation of Lysate for Western Blotting

Culture supernatants were diluted 1:2 in whole podocyte medium (as described above) and applied to the differentiated podocytes. Following an incubation period of between 5 and 60 minutes the cells were lysed and protein was extracted using a Triton-X based extraction buffer (Trizma 50mM, EDTA 1.23mM, NaCl 120mM, NaF 50mM, Sodium Beta Glycerophosphate 40mM, Benzamidine 1.33mM, Triton X 16mM) supplemented with protease and phosphatase inhibitor cocktails (Sigma P1860, P0044 and P5726). The culture was scraped, and the lysate centrifuged to remove any membrane fragments and other contaminants. The cell culture lysate was snap frozen in liquid nitrogen. The lysates were run on 10% acrylamide gels unless otherwise indicated.

Scratch Assay

Podocytes were cultured in 6 well plates. Scratch assays were performed once the podocytes had spent 14 days at the non-permissive temperature of 37°C. In brief, the media was aspirated, and a mechanical wound was inflicted by scratching the monolayer with a pipette tip. The podocytes were then washed in PBS twice to remove any debris and pro-migratory factors. Then the diluted Th0/Th17 cell culture supernatant was added to the wells and the scratch area was imaged after 0 (control) and 12 hours using a Leica DMIRB microscope and a Zeiss Axiocam ERc 5S camera. The area of the clear zone was measured over time and podocyte migration assessed by reduction in the area, indicating more motile

cells. The experiment was repeated 8 times with different batches of supernatant. Each repeat had at least 3 but up to 6 replicates.

Inhibitors

The p38 MAPK inhibitor SB203580 (Sigma) was used at a dose of 10µM [2], the JNK inhibitor SP600125 (Tocris), was used at a concentration of 1µM [3]. The PAR-1 Receptor antagonist Vorapaxar (ADV465750877 Sigma) was used at a concentration of 15µM The PAR-1 inhibitor FR171113 (#SML0028 SIGMA) was used at a concentration of 15µM. Th17 culture supernatants were pre-treated with the inhibitors for 30 minutes before being added to the podocytes.

ELISA

The IL-23 ELISA (88-7237-22, eBioscience) was performed according to the manufacturer's protocol.

PAR-1 Agonist

The PAR-1 agonist (PAR-3931-PI Peptides International) is a short peptide that contains the sequence of the tethered ligand. Podocytes were treated with 15µM for up to 60 minutes.

Viability Assay

The CellTiter 96® AQueous One Solution Cell Proliferation (MTS) Assay (Promega G3582) was performed to assess cell viability. Podocytes were cultured for 14 days at the non-permissive temperature of 37°C in 96 well plates. They were then treated for 24h before MTS Tetrazolium (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)) was added to the wells. Viable cells metabolise this substrate to produce formazan which is read at an absorbance 490nm using Dynex Technologies Opsys MR plate reader).

Graphs and Statistics

Graphs show mean and standard error of the mean (SEM) unless otherwise indicated and were compiled and analysed (see figure legends) using Graphpad Prism (version 5.01). Asterix "*", will be used to denote level of significance within a graph. For p=<0.05, "*", for p=<0.01 "**" and for p=<0.001 "***". When different sets of comparisons are being made within the same graph the hash "#" will be used in place of the asterisk. Interactions were assessed using Bonferroni's multiple comparison test unless otherwise stated.

Results

Th17 Cell Culture Supernatant and patient disease plasma? Stimulates p38 MAPK and JNK Signalling Pathways

The addition of Th17 cell culture supernatant (from healthy volunteers) to podocytes *in vitro* significantly stimulated the stress response kinases p38 MAPK and JNK in podocytes at 30 mins and 15 mins respectively (**Figure 1**). Neither the Th0 nor Th17 cell culture supernatant treatments had a significant effect on podocyte viability.

Paxillin is Phosphorylated at the JNK Specific Site but not the p38 MAPK Specific Site

The p38 MAPK and JNK signalling pathways converge downstream; p38 MAPK and JNK have specific target phosphorylation sites on paxillin s85 and s178 respectively. Paxillin is a signalling platform that is localised to focal adhesion sites [4]. It plays an important role in organising the cytoskeleton and adhesion changes that are necessary for cellular migration. We therefore examined whether Th0 and Th17 supernatants could phosphorylate paxillin. This revealed that the s178 phosphorylation site was phosphorylated at 15, 30 and 45 minutes following the addition of Th17 cell culture supernatant (**Figure 2**). As this is coincident with the phosphorylation of JNK (**Figure 1**) it is feasible that JNK is phosphorylating paxillin at s178 in podocytes and that this is providing a pro-migratory signal [9]. The s85 site was only minimally phosphorylated in podocytes treated with either Th0 or Th17 supernatant (**Figure 2**). ~~Hence, p38 MAPK signalling is not sufficient to lead to the phosphorylation of paxillin at the p38 MAPK specific site.~~ It is unclear why Paxillin is not phosphorylated by p38 MAPK at serine 85. Given the clear stimulation of p38 MAPK shown in Figure 1, it was certainly expected that s85 phosphorylation on Paxillin would be detected.

Th17 Cell Culture Supernatant Significantly Increases Podocyte Motility via JNK Signalling

Foot process effacement is an early response of the podocyte to injury and is a key event in the development of proteinuria. Podocyte motility *in vitro* was assessed using a wound healing assay (**Figure 3A**) to determine whether exposure to Th17 cell supernatant rendered podocytes more motile. Collated data from scratch assay experiments using Th0/Th17 supernatants from 8 seperate donors shows that Th17 supernatant treatment significantly increased the motility of podocytes relative to Th0 treatment (**Figure 3B**). To determine the

role of JNK and p38 MAPK in this process, podocytes were treated with Th17/Th0 culture supernatants that had been pre-treated (30mins) with either a JNK inhibitor or a p38 MAPK inhibitor. The JNK inhibitor blocked the motility response to Th17 cell culture supernatant (**Figure 3C**). This observation suggests that the JNK signalling pathway is a crucial step in mediating the response to the secreted factor and generating a motile phenotype.

Neither IL-17 nor Constituents of the Th17 Medium are Responsible for the Increase in Motility

Th17 cells produce a variety of cytokines including IL-17A making this a candidate effector protein present in the Th17 cell culture supernatant. However, IL-17A alone had no significant effect on podocyte motility at any of the doses tested (0.1 to 100ng/ml) (**Figure 4A**). We also tested the effect on podocyte motility of each single cytokine used to polarise the Th17 cells (IL-1B, IL-2, IL-6 and IL-23). IL-23 (used at 10ng/ml) was the only cytokine to significantly increase podocyte motility (**Figure 4B**). To interrogate the IL-23 effect further, we sought to investigate whether this was the factor in the Th17 supernatant causing the increased motility seen in Figure 4. However, since the supernatants are diluted before addition to podocytes, we first measured the actual concentration of IL-23 in the diluted Th17 supernatants (**Figure 4C**) and then added this concentration (0.7ng/ml) to the podocytes. However, at this lower dose, IL-23 had no significant effect on podocyte motility (**Figure 4D**).

PAR-1 Agonist Treatment Elicits the Same Response as Th17 Cell Culture Supernatant Treatment

Previous work has suggested that the circulating factor implicated in non-genetic forms of NS signals via PAR-1 [8]. Podocytes were treated with a PAR-1 agonist to ascertain to what extent the signalling and behavioural responses to the Th17 cell culture supernatant could be replicated. The signalling responses of the podocytes to PAR-1 agonist treatment mimicked the response to Th17 cell culture supernatant treatment: pJNK, p38 MAPK and pPaxillin s178 pathways were all stimulated (**Figure 5A-C**), while Paxillin s85 was unaffected (**Figure 5D**). This indicates that the JNK signalling pathway and PAR-1 activation mimic the hyper-motile phenotype and altered signalling observed in podocytes treated with Th17 cell supernatants.

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To further investigate the role of the PAR-1 receptor in response to Th0/Th17 cell culture supernatant, podocytes were treated with the supernatants along with the intracellularly acting PAR-1 inhibitor FR171113 and the FDA approved PAR-1 antagonist Vorapaxar. The significant increase in pJNK, p38 MAPK and pPaxillin s178 and VASP was seen in response to the addition of Th17 cell culture supernatant relative to Th0 cell culture supernatant, and FR171113 significantly reduced the phosphorylation of VASP, p38 MAPK and JNK in response to Th17 cell culture supernatant (**Figure 6**). Surprisingly Vorapaxar treatment had no significant effect on the signalling response. Figures 6A-6D show the densitometry of the targeted proteins with Figure 6E showing representative blots.

Discussion

This work suggests that Th17 cells release a hitherto unknown factor ~~that activates the PAR-1 receptor and~~ stimulates JNK, MAPK Paxillin and, importantly, VASP signalling pathways, inducing deleterious effects on podocyte morphology and function akin to that which occurs in NS.

It is envisaged that a subset of pathogenic Th17 cells expand and release a hitherto unknown serine protease that could possibly cleave the PAR-1 receptor on the podocyte. This induces a series of pathological signalling events which result in foot process effacement, increased podocyte motility and proteinuria. Such a situation is consistent with current thinking on steroid sensitive, steroid resistant and steroid dependant Nephrotic Syndrome.

A role for Th17 cells in nephrotic syndrome is becoming increasingly clear. IL-17 has been implicated in causing podocyte damaged, indeed blocking IL-17, which is predominantly secreted by Th17 cells, improves albuminuria in a model of Diabetic Nephropathy [23] [15].

The glomerular filtration barrier restricts passage of proteins and macromolecules based on their size and charge. Molecules such as insulin (5 kDa) pass freely through the barrier. Molecules as large as Myoglobin (16.9 kDa) pass through relatively uninhibited. Only molecules larger than 60 kDa are restricted to a great extent. Hence a serine protease with a molecular weight lower than 17 kDa or so would be able to pass through the filtration barrier and stimulate signalling in the podocyte [6].

We have interrogated signalling pathways and podocyte motility *in vitro*, as a proxy for foot process effacement *in vivo,* and shown that Th17 cell culture supernatant significantly increased podocyte motility and induced clearly defined signalling pathways in keeping with PAR-1 activation. These data suggest that a soluble mediator generated by Th17 cells affects podocytes in a manner deleterious to barrier function within the glomerulus and is a novel candidate mechanism of NS pathogenesis. However, there is yet to be a consensus on the role of immune mediators in NS. Nonetheless, the active phase of minimal change NS has been linked to an increase in IL-17A producing Th17 cells and an increase in Th17 cells and their markers has also been reported during active disease (12, 13). In addition, an increase in the expression of other pro-inflammatory markers, such as tumor necrosis factor alpha (TNF-α) and IL-6 during proteinuria has also been reported (14).

Th17 cell culture supernatant significantly stimulated JNK and p38 MAPK signalling pathways in podocytes compared to Th0 supernatant. Although co-stimulation of these pathways can indicate cell stress, a viability assay showed that neither Th0 nor Th17 cell culture supernatant treatment had a deleterious effect on podocyte viability. The p38 MAPK signalling pathway is known to be stimulated in response to podocyte injury [14] and Koshikawa *et al* showed increased phosphorylated p38 MAPK in podocyte nuclei in biopsies from minimal change nephropathy, membranous nephropathy and focal segmental glomerulosclerosis patients relative to healthy controls. Additionally they found that marked p38 MAPK phosphorylation preceded proteinuria in experimental models of NS and that the proteinuria could be blocked by the administration of a p38 MAPK inhibitor [14]. Consistent with this, we observed that administration of Th17 cell culture supernatant to podocytes *in vitro* significantly increased p38 MAPK signalling 30 minutes post-treatment relative to Th0 treatment.

The p38 MAPK and JNK pathways converge downstream at Paxillin and mediate phosphorylation of paxillin at serine residues 85 and 178 respectively. This has been associated with altered cell motility as S85A mutant neuronal cells display neurite outgrowth in response to nerve growth factor administration [10] while in S178A mutant epithelial cells and keratocytes cell migration is inhibited [9]. This suggests that paxillin plays a key role in the coordination of migratory responses. However, we showed that podocytes treated with Th17 cell culture supernatant only phosphorylated paxillin at serine 178 and not at serine 85 suggesting that the JNK signalling response was more important for generating the increased motility.

JNK was significantly phosphorylated by 15 minutes following Th17 cell culture supernatant treatment and by 15 minutes JNK had phosphorylated its target site on Paxillin (S178). This phosphorylation event has been associated with increased cell migration in epithelial cells and could therefore be driving the increased podocyte motility seen in response to Th17 cell culture supernatant treatment. Indeed, when podocytes were treated with a JNK inhibitor there was a significant reduction in Th17 induced podocyte motility. This supports the notion that the JNK pathway is key to generating the motile phenotype in podocytes.

Furthermore, this study aligns Th17 cells with our previous observation that the circulating factor implicated in relapsing forms of NS signals via the protease-activated-receptor PAR-1 [8]. In this study podocytes treated with a PAR-1 agonist mimicked the response to Th17 cell culture supernatant treatment consistent with PAR-1 being involved in the Th17 supernatant mediated podocyte changes, and we extended our previous results with patient relapse/remission plasma to show the same MAPK/JNK/paxillin signalling. Accordingly, direct inhibition of the PAR-1 receptor using FR171113 significantly reduced the signalling response to Th17 cell culture supernatant and patient plasma treatment. Although further study is needed, FR171113 appears to be a specific inhibitor of PAR-1 with no apparent side-effects [12].

Vorapaxar had no significant effect on the signalling response of the podocyte to Th17 cell culture supernatant treatment (Figure 6). This was somewhat surprising given that Vorapaxar is a commercially available, FDA approved drug used to reduce the risk of stroke or myocardial infarction, where it targets the PAR-1 receptor in platelets. Only one dose of vorapaxar was

tested owing to the scarcity of the Th0 and Th17 cell culture supernatants. Ideally more doses of the antagonist would be tested. Vorapaxar competes with the tethered ligand for the binding of the active site. Therefore large concentrations of vorapaxar may be necessary in order to compete with the very high localised concentration of the tethered ligand [24]. Vorapaxar is an orthosteric inhibitor that binds to the ligand binding site (active site) of the PAR-1 receptor. As such this inhibitor blocks the binding of the tethered ligand and hence auto-activation of the receptor. FR171113 is an intracellular allosteric inhibitor. FR171113 appears to be a selective inhibitor given its effects on clotting in guinea pigs: FR171113 inhibits thrombin-mediated platelet aggregation and was not seen to have any adverse effects [11].

The ability of FR171113, and inability of Vorapaxar, to block PAR-1 signalling could be informative as to the identity and mechanism of the circulating factor. As described, we may not have seen inhibition of signalling since we are not using a sufficient concentration of vorapaxar. Alternatively, the unknown factor(s) in the Th17 cell culture supernatant could be cleaving the extracellular domain of PAR-1 at an alternative site. This would cause what may remain of the tethered ligand to bind the receptor elsewhere than the active site.

FR171113, however, binds allosterically to the PAR-1 receptor and blocks the conformational change in the activated form of the receptor. In this way FR171113 can block the activation of the receptor independent of the way in which the receptor has been activated, whereas vorapaxar can only inhibit classical activation of PAR-1 by the tethered ligand binding the active site.

Importantly, the response of podocytes to Th17 cell supernatant was neither mediated by the reagents added to the media of our human Th17 cells cultures, nor by IL-17A itself. This suggests that another soluble mediator produced by Th17 cells and absent in Th0 cell supernatants is mediating the observed effects on podocyte signalling and motility. Our inhibitor studies suggest that this factor may be a protease that signals via the JNK pathway to induce a state of podocyte hypermotility.

A bias towards the Th17 phenotype in the Treg/Th17 axis has been shown in Minimal Change Nephrotic Syndrome [16].

Although far from conclusive, the inhibitor studies presented here suggest a possible role for the PAR-1 receptor in the signal transduction pathway between the circulating factor and podocyte damage.

If Th17 cells play a role in the pathogenesis of non-genetic NS, they may also affect response to treatment. Given that GCs remain the standard first-line therapy for NS, and that subsets of Th17 cells have been recently reported across a range of conditions to be candidate mediators of GC resistance, this has potential translational relevance for patients with GC refractory disease. In summary, we have demonstrated for the first time that human Th17 cells are able to directly cause podocyte damage via signalling pathways recently described to be activated by NS patient plasma [8]. We consequently propose that Th17 cells are a candidate mediator of NS pathogenesis with the potential to also influence the efficacy of GCs administered in the treatment of active disease.

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Figure Legends

Figure 1: Podocyte Signalling Response to Th17 Cell Culture Supernatant. The addition of Th17 cell culture supernatant to podocytes elicits a significant response in both **A)** p38 MAPK and **B)** pJNK signalling pathways relative to Th0 cell culture supernatant treatment. Statistical significance measured using One-Way ANOVA and a post-hoc Bonferroni's Multiple Comparison test.

Figure 2: JNK Phosphorylation induced by Th17 Cell Culture Supernatant Leads to Phosphorylation of Paxillin in Podocytes. Both p38 MAPK and JNK have specific phosphorylation sites on Paxillin: at serines 85 and 178 respectively. Only the JNK specific site at serine 178 is phosphorylated. Representative of two blots.

Figure 3: Th17 Cell Culture Supernatant Significantly Increases Podocyte Motility Relative to Th0 Cell Culture Supernatant. A.) Podocytes treated with Th17 cell culture supernatant were significantly more motile than those treated with Th0 cell culture supernatant (n=8, p=0.0007 Unpaired T Test). The area of the clear zone is measured in pixels. The area at 12h is normalised to 0h. Units are arbitrary. **B).** To establish the importance of the signalling events detected by western blot, podocytes were treated with Th17 supernatant and inhibitors against p38 MAPK and JNK. Motility was measured in these podocytes by using a wound healing assay. JNK inhibition significantly reduced the Th17 mediated increase in podocyte motility. Statistical significance measured using One-Way ANOVA and a post-hoc Bonferroni's Multiple Comparison test. The area of the clear zone is measured in pixels. The area at 12h is normalised to 0h. Units are arbitrary. **C.)** A typical set of images that are taken to measure podocyte motility. The area of the clear zone 12 hours post scratch is expressed as a decimal of the area at 0h. Scale bar in images represents 500μM.

Figure 4: IL-23 can Significantly Increase Podocyte Motility Though not at the Dose Present Initially in the Th17 Cell Culture Supernatant. **A.)** IL-17 had no significant effect on podocyte motility at any

of the doses tested (n=3). **B.)** IL-23 is the only supplementary cytokine that is capable of significantly increasing podocyte motility (6 replicates p=0.0041 Unpaired T Test). However, the podocytes were treated with 10ng/ml dose of IL-23, which is the concentration at which IL-23 is added to the Th17 cell culture medium. By the time the supernatant has been applied to the podocytes the medium has been in the presence of Th17 cells for 48 hours and through one freeze-thaw cycle. **C.)** Therefore, an ELISA was performed in order to determine the concentration of IL-23 that remains in the Th17 cell culture supernatant. There is 0.7mg/ml of IL-23 in the Th17 cell culture supernatant. **D.)** At this dose IL-23 has no significant effect on podocyte motility.

Figure 5: PAR-1 Agonist Signalling Response Mimics the Th17 Cell Culture Supernatant Response. Conditionally immortalised podocytes were treated with 15μM PAR-1 agonist for the indicated time course. The control lysate was generated from untreated podocytes.Data shown is combined densitometry of replicate blots normalised to a beta-actin load control (not shown) with a representative blot beneath. **A.)** p-JNK signalling. **B.)** p-38 MAPK signalling. **C.)** p Paxillin s178. **D.)** P Paxillin s85. The signalling responses for each were dynamically like those elicited by Th17 cell culture supernatant treatment. Data represents 4 independent experiments. Statistical significance measured using One-Way ANOVA and a post-hoc Bonferroni's Multiple Comparison test.

Figure 6: PAR-1 Inhibition Blocks the Signature Th17 Cell Culture Supernatant Response. Th17 cell culture supernatant treatment of podocytes significantly increased the phosphorylation of **A.)** p-JNK, **B.)** p38 MAPK, **C.)** p Paxillin s178, **D.)** p-VASP 157. Conversely, inhibition of PAR-1 by FR171113 significantly reduced each Th17 response (Densitometry based on 4 blots, One-Way ANOVA and a post-hoc Bonferroni Multiple Comparison Test). **E.)** Representative blots of proteins studied.